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Volatile Anesthetics Transiently Disrupt Neuronal Development in Neonatal Rats

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ABSTRACT

Volatile anesthetics can cause neuronal and glial toxicity in the developing mammalian brain, as well as long-term defects in learning and memory. The goals of this study were to compare anesthetics using a clinically relevant exposure paradigm, and to assess the anesthetic effects on hippocampal development and behavior. Our hypothesis was that volatile anesthetics disrupt hippocampal development, causing neurobehavioral defects later in life. Bromodeoxyuridine (BrdU) was administered to rats on postnatal day (P)1, and the rats were exposed to volatile anesthetics (isoflurane, sevoflurane, or desflurane) for 2 h on P2. On days P7 and P14, the BrdU-labeled cells were quantified in the hippocampal dentate gyrus using immunohistochemical assays and fluorescent microscopy. Caspase-3 positive cells were quantified on P2 to evaluate apoptosis. The remaining animals underwent behavioral testing at ages 6 weeks and 6 months, using the Morris Water Maze. Significantly fewer BrdU-positive cells were detected in the hippocampal dentate gyrus in both isoflurane and desflurane-treated animals compared with controls at P7, but there were no changes in cell numbers after sevoflurane exposure. Cell counts for all three anesthetics compared with controls were equivalent at P14. Isoflurane or desflurane exposure yielded slight differences in the behavioral tests at 6 weeks, but no differences at 6 months post-exposure. We conclude that a single 2-h exposure at P2 to either isoflurane or desflurane causes a transient disruption of hippocampal neuronal development with no significant detectable long-term effects on learning and memory, whereas the same exposure to sevoflurane has no effects.

Key words: anesthesia; neonatal; development; neurogenesis; neurotoxicity; learning and memory.

Children receive general anesthesia frequently for both surgical and nonsurgical procedures, and concern has been raised that the anesthetics themselves may be harmful to the developing brain. Human studies have focused on various outcomes, including rates of behavioral disorders (DiMaggio *et al.*, 2009, 2011; Flick *et al.*, 2011; Ing *et al.*, 2012; Kalkman *et al.*, 2009), learning disabilities (Flick *et al.*, 2011; Wilder *et al.*, 2009), ADHD (Sprung *et al.*, 2012), language ability (Ing *et al.*, 2012), and cognitive performance (Bartels *et al.*, 2009; Flick *et al.*, 2011; Ing *et al.*, 2012). While these studies have produced mixed results, most suggested that children who underwent multiple anesthetics had a greater likelihood of being diagnosed with a behavioral or cognitive problem (DiMaggio *et al.*, 2009, 2011; Flick *et al.*, 2011; Ing *et al.*, 2012; Kalkman *et al.*, 2009; Sprung *et al.*, 2012; Wilder *et al.*, 2009).

Animal studies have shown that exposure to volatile anesthetics alone can cause neuronal and glial toxicity in the developing mammalian brain, as well as defects in learning, memory, and behavior. These toxic effects could be due to disruption of different developmental processes. For example, several groups have studied apoptosis rates following an anesthetic exposure, and have shown more apoptosis in the

brains of exposed animals (Brambrink et al., 2010, 2012; Istaphanous et al., 2011; Jevtovic-Todorovic et al., 2003; Liang et al., 2010; Yang et al., 2014; Zou et al., 2011). Furthermore, anesthetic exposure has been shown to affect neurogenesis (Stratmann et al., 2009b; Zhu et al., 2010) and neuroinflammation (Shen et al., 2013) in the developing rat brain. A combination of general anesthetics was also shown to impair mitochondrial morphogenesis and synaptic transmission during rat brain development (Sanchez et al., 2011). Cell culture studies have shown that anesthetic exposure impairs axonal guidance cues (Mintz et al., 2013) and disrupts cytoskeletal structure in astroglia (Lunardi et al., 2011).

Behavioral studies in animals have produced mixed results. Some groups have shown that rats have impaired learning and memory in various behavioral tests after anesthetic exposure (Jevtovic-Todorovic et al., 2003; Shen et al., 2013; Stratmann et al., 2009b; Zhu et al., 2010). However, others have reported that even though exposed animals had increased apoptosis, there were no differences in behavior after anesthesia (Liang et al., 2010; Yang et al., 2014).

These studies have given us important information about how anesthetics may disrupt brain development, but there are many developmental processes that have not been studied. The sequence of events for brain development includes proliferation, migration, differentiation, synaptogenesis, myelination, and apoptosis (Altman and Bayer, 1975; Angevine, 1975; Bayer et al., 1993). Neuronal proliferation, migration and differentiation are important early developmental processes required for neurons to form their correct synaptic connections. In the developing rat brain, these events occur prenatally in most brain regions; however, in the dentate gyrus of the hippocampus, neuronal proliferation, migration and differentiation continues postnatally (Altman and Bayer, 1975; Angevine, 1975; Bayer et al., 1993). In the neonatal rat, immature neurons proliferate in the subgranular zone (SGZ) of the hippocampus and then migrate along radial glial cells to their final positions in the granular layer of the dentate gyrus (Altman and Bayer, 1975; Angevine, 1975; Bayer et al., 1993; Corbin et al., 2008; Eckenhoff and Rakic, 1984; Rickmann et al., 1987). Given that prior studies have focused on synaptogenesis and apoptosis at postnatal day (P) 7, we believe it is important to examine the earlier events of neuronal proliferation and migration.

Prior animal studies have used combinations of anesthetics (Jevtovic-Todorovic et al., 2003; Sanchez et al., 2011; Zou et al., 2011), anesthetics at relatively high doses (Shen et al., 2013), or long anesthetic exposures (Brambrink et al., 2012; Istaphanous et al., 2011; Liang et al., 2010; Shih et al., 2012; Stratmann et al., 2009b), potentially introducing physiologic changes (such as hypercarbia) not present in pediatric patients. Furthermore, most prior studies have exposed rodents to anesthetics on P7 (Istaphanous et al., 2011; Jevtovic-Todorovic et al., 2003; Liang et al., 2010; Shen et al., 2013; Stratmann et al., 2009b; Yang et al., 2014; Zhu et al., 2010), and have provided invaluable information regarding the effects of anesthetic exposure at this very important time point in rodent brain development. Children of all ages receive anesthesia, so we must continue to study the effects of anesthetics at multiple developmental stages in rats as well. We chose to expose rats to anesthetics on P2 in order to gain new information about how anesthetics affect neurodevelopment at this earlier time point. We also used lower concentrations of anesthetics and shorter exposures than many other studies to more closely mimic clinical practice. We compared three clinically used anesthetics (isoflurane, sevoflurane, and desflurane) to identify differences in their toxicity profiles. We hypothesized that volatile anesthetics would disrupt hippocampal development, causing neurobehavioral defects later in life.

MATERIALS AND METHODS

Animal care. The animal protocol was approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC) and all rats were treated in strict accordance with APS/NIH guidelines. Timed pregnant female Sprague–Dawley rats (Charles River, Horsham, Pennsylvania) and litters were housed at the animal facilities at the University of Pennsylvania. The birth date of the pups was considered to be P0.

BrdU injections. On P1, all pups, except the group euthanized at P2 for caspase-3 immunohistochemistry, were given two intraperitoneal (i.p.) injections of 5-bromo-2′-deoxyuridine (BrdU) (Sigma–Aldrich B5002, St. Louis, Missouri) 50 mg/kg, 4 h apart. The average weight of rats on P1 was 7.23 g. BrdU solution (5 mg/ml) was made using sterile saline and NaOH for pH neutralization to 7.4, filtered using a 0.2-μm filter in a sterile hood, and stored at −20°C. The animals were immediately returned to the cage with the mother after each injection, and resumed normal feeding behavior.

Anesthetic exposures. On P2, rat pups from a total of 18 litters were randomly divided into treatment and control groups and anesthetized with isoflurane, sevoflurane, or desflurane for 2 h. Sex was not determined at this age. As previously described (Liang et al., 2010), an anesthetic chamber setup was used. The Plexiglas anesthetic and control chambers were placed in a fume hood and kept partially submerged in a 44 °C ± 1°C water bath, resulting in the air temperature just above the chamber floor being 30 °C ± 1°C, which has been shown to maintain physiologic temperatures in P7 mice. Controls and anesthetized groups received heated and humidified 30% oxygen balanced with nitrogen at a total oxygen plus air flow rate of 4 L/min for the duration of the experiment, with all groups having maternal separation in the Plexigas chambers for the duration of the 2-h exposure. Continuous chamber anesthetic gas, oxygen, and carbon dioxide concentrations were measured with gas analyzers (Ohmeda 5330 Agent Monitor, Ohmeda, Louisville, Colorado; Capnomac Ultima, Datex–Engstrom, Helsinki, Finland) during the 2-h exposures for control and experimental groups. Carbon dioxide concentrations remained at 0% for all experiments, as is expected for this experimental setup using a relatively high gas flow rate and large chamber compared with animal size. This setup prevents accumulation of exhaled CO_2 and effectively maintains a steady state of all gases in the chamber.

Postnatal day 2 pups in the treatment groups were induced (determined by loss of righting reflex and loss of tail clamp response in >50% of the animals) with either 3.5% isoflurane for 5 min and maintained at 1.5% isoflurane for the remainder of the exposure, 5% sevoflurane and maintained at 2.5%, or 12% desflurane and maintained at 7%. The concentrations chosen for maintenance were based on prior work in P7 rats showing that the minimum alveolar concentration (MAC) at the 2-h time point during an anesthetic exposure was 2.0% for isoflurane, 3.3% for sevoflurane, and 9.2% for desflurane (Stratmann and Alvi, 2011). These doses are also supported by prior work in P2 rats showing that the MAC for isoflurane is 1.9%, and the MAC for sevoflurane is 3.3% (Orliaguet et al., 2001), which correlates exactly with the data for P7 rats. We chose to use an anesthetic

concentration equivalent to 0.75 MAC, which is clinically relevant in the neonatal age group, and also results in very low mortality. During the exposures, animals were constantly observed for changes in skin color and chamber oxygen/carbon dioxide concentrations. The respiratory rate and response to tail pinch were monitored every 30 min by briefly opening the chamber. The anesthetic concentration and air/oxygen flows were then briefly increased until the concentration in the chamber returned to 0.75 MAC. In addition, at the end of the 2-h anesthetic exposures, while still under anesthesia, a subset of animals was used to obtain arterial blood for determination of arterial blood gas (ABG) data via blind direct left ventricular puncture, a terminal procedure commonly used in neonatal rats (Jevtovic-Todorovic *et al.*, 2003; Liang *et al.*, 2010; Shih *et al.*, 2012; Stratmann *et al.*, 2009a,b). Blood was collected in heparinized 1-cc syringes and immediately analyzed in a blood gas analyzer (Siemens RAPIDLab 248, Malvern, Pennsylvania). We obtained ABGs from control rat pups by anesthetizing them with isoflurane for 15–20 min (until no response to tail clamp) prior to this procedure. Bright red blood was determined to be arterial blood, but a limitation of this blind procedure is that the source of blood cannot be verified. There were no statistically significant differences in pH, partial pressure of carbon dioxide (pCO_2), or partial pressure of oxygen (pO_2) for each anesthetic group compared with control (Table 1). The remaining animals were removed one at a time from the anesthetic chamber and, prior to emergence from anesthesia, immediately microtattooed with one dot on the paw or base of tail (in <15 s) with Ketchum animal tattoo paste (Braintree Scientific, Braintree, Massachusetts) using an Aramis microtattoo kit (Braintree Scientific) with a 26 1/2 G needle for later identification. The rat pups for the caspase-3 study were not tattooed because they were immediately euthanized and could be identified with a sharpie pen. Microtattoo is the least noxious marking technique, with microtattooed animals having significantly lower heart rate and blood pressure than animals that were given ear tattoos or ear notching (Kasanen *et al.*, 2011). Furthermore, the more traumatic practice of tail biopsy for genotyping has no effect on pain behaviors (Morales and Gereau, 2009). These studies suggest that the less invasive technique of microtattooing likely had no effect on behavior, apoptosis, or cell counts in our study.

After the 2-h exposures, the animals were removed from the anesthetic chamber and placed on disposable chucks over a heating pad (surface temperature 37°C) to recover. Mortality from the exposures were: control 0%, isoflurane 3.8%, sevoflurane 5.3%, and desflurane 4.8% (not statistically significant, $P = .35$). These are very low when compared with similar studies reporting 21%–25% mortality after a 4-h anesthetic exposure (Istaphanous *et al.*, 2011; Stratmann *et al.*, 2009a). We hypothesized that death occurred in this small subset of animals because of respiratory depression leading to hypoxia and/or hypercarbia, though we did not perform any analysis to test this hypothesis. When the animals had fully recovered from anesthesia as evidenced by coordinated locomotion (after approximately 15 min), they were returned to cages with their mothers and resumed normal feeding behavior.

Tissue preparation and immunohistochemistry. Rats were euthanized on P2, P7, or P14, or after the second round of behavioral studies at 7 months of age. The animals were deeply anesthetized with the anesthetic corresponding to their exposure and perfused through the left cardiac ventricle with phosphate buffered saline (PBS, pH 7.4) with right atrial exsanguination, followed by perfusion with cold 4% paraformaldehyde. The brains were removed and post-fixed in 4% paraformaldehyde at 4°C for 1–3 days. The brains were then dehydrated, paraffin-fixed, and embedded in paraffin blocks for sectioning. Serial coronal sections were cut using a microtome set to 8 μm and adhered to glass slides.

Newly formed cells in the subgranular zone (SGZ) of the dentate gyrus differentiate and migrate to the granule cell layer over the course of days to weeks (Angevine, 1975). Therefore, in order to study the effects of anesthetic exposures on neuronal proliferation and migration in the hippocampal dentate gyrus, immunohistochemistry was performed on P2 (caspase-3 only), P7, and P14 brain sections, as described previously (Peng *et al.*, 2014; Zhu *et al.*, 2010), with minor modifications as follows. The sections were deparaffinized in xylenes, then rehydrated using serially decreasing ethanol concentrations. Antigen retrieval was carried out in a decloaking chamber (Biocare Medical DC1509, Concord, California) with the tissue submerged in sodium citrate buffer 10 mM, pH 6.0, for 2 min with a maximum pressure of 30 psi and maximum temperature of 125°C, followed by depressurization and cooling. Blocking buffer (0.1 M PBS, 0.3% Triton X-100, 2% goat serum) was applied to the tissue for 60 min. The sections were incubated with primary antibodies overnight at 4°C (Table 2), followed by incubation with the secondary antibody (Table 2) for 2 h at room temperature. All sections were coverslipped using Prolong Gold Anti-Fade Reagent (Life Technologies P36930, Carlsbad, California) mounting medium, except for the caspase-stained slides, which were coverslipped using ProLong Gold Anti-fade Reagent with DAPI (Life Technologies P36931) mounting medium.

Imaging. Quantitative imaging was performed by an investigator blinded to the treatment groups using sections chosen from each brain starting at the anterior hippocampus, corresponding to figure 57 (page 61) of the Atlas of the Neonatal Brain (Ramachandra and Subramanian, 2011), and using every 10th section thereafter for a total of five coronal sections per brain, allowing us to capture cells throughout the dentate gyrus. Fluorescence microscopy was performed using an Olympus BX41 microscope equipped with a digital camera (QImaging, Surrey, British Columbia, Canada) and computer running IPLab software (Scanalytics, Scientific Instrument Company, Campbell, California) for image capture.

TABLE 1. Arterial Blood Gas Analysis

	Control (n=6)	Desflurane (n=4)	Sevoflurane (n=8)	Isoflurane (n=6)	P value
pH	7.31±0.02	7.32±0.02	7.21±0.03	7.20±0.04	0.0562
pCO_2	65.22±4.29	49.35±7.03	60.09±6.05	71.00±6.05	0.3025
pO_2	62.48±3.95	57.23±3.14	74.25±13.43	52.35±4.60	0.5093

Data are expressed as means ± SEM.
P values for each blood gas parameter from one-way ANOVA of anesthetic exposures compared with control.
pCO_2, partial pressure of carbon dioxide; pO_2, partial pressure of oxygen.

TABLE 2. Antibodies

Raised against	Supplier (ID)	Raised in	Dilution	Corresponding secondary antibody
BrdU	Serotec (MCA2060)	Rat, monoclonal	1:1000	Alexa Fluor 594 goat anti-rat IgG (A11007)
NeuN	Millipore (MAB377)	Mouse, monoclonal	1:500	Alexa Fluor 488 goat anti-mouse IgG *highly cross adsorbed* (A11029)
Tuj1	Covance/BioLegend (MMS-435P)	Mouse, monoclonal	1:500	Alexa Fluor 488 goat anti-mouse IgG *highly cross adsorbed* (A11029)
Cleaved caspase-3	Cell Signaling (Asp175) (5A1E)	Rabbit, monoclonal	1:500	Alexa Fluor 594 goat anti-rabbit IgG *highly cross adsorbed* (A11037)

BrdU, bromodeoxyuridine; NeuN, neuronal nuclear antigen; Tuj1, neuron-specific class III beta-tubulin.

For BrdU immunohistochemistry, the rat pup brains from each treatment group at P7 and P14 were examined using a 10× objective and imaged for BrdU and NeuN (neuronal nuclear antigen), or BrdU and Tuj1. While both neuronal markers, NeuN and Tuj1 (neuron-specific class III beta-tubulin), provided adequate staining of neurons to identify the structure and layers of the hippocampus in all sections, neither showed definitive co-localization with BrdU at P7 or P14 for quantitative purposes. It is possible that these BrdU-positive cells were too young to adequately express either neuronal marker. The regions of interest in the dentate gyrus (outer and inner halves of the granular layer (GCL), and subgranular zone (SGZ, including the hilus) were first traced on each image using Adobe Photoshop software (Adobe Systems, San Jose, California) using only the filter corresponding to the NeuN or Tuj1 staining, both of which provided an anatomical map for neuronal layers and also kept the investigator blinded to the location of BrdU-positive cells. Then, the number of BrdU-positive cells in each region per unit area were counted using only the filter for BrdU staining with ImageJ software (NIH, http://imagej.nih.gov/ij/index.html, last accessed on 30 August 2016). The mean number of BrdU-positive cells was calculated from five to six brain sections for each animal, $n = 3$ animals for each group at both ages.

In summary, we injected P1 rats with BrdU to label cells formed on P1, and the rat pups were exposed to volatile anesthetics on P2. BrdU-positive cells were counted at two time points (P7 and P14) in the dentate gyrus, with representative images shown in Figure 1.

In order to examine whether differences in the BrdU-positive cell counts may have been due to apoptosis caused by the anesthetic exposures, we performed activated caspase-3 immunohistochemistry on hippocampal sections obtained from P2 rats sacrificed immediately after the 2-h anesthetic exposures. For caspase-3 immunohistochemistry, P2 caspase-stained slides from each treatment group were imaged at 20×, and the number of caspase-3 positive cells per area were counted as described above for BrdU, first tracing the combined GCL and SGZ under the DAPI filter and then using ImageJ software (NIH, http://imagej.nih.gov/ij/index.html, last accessed on 30 August 2016) to count the caspase-3 positive cells. The mean number of caspase-3 positive cells was calculated from five to six brain sections for each animal, $n = 5$ animals for each group.

Morris water maze. Rat pups were allowed to mature for behavioral testing in the Morris water maze (MWM) at 6 weeks and 6 months of age ($n = 12$). A total of 48 rats were used for the behavioral experiments, including both male (M) and female (F) rats, randomly divided among the experimental groups. The sex of the rats per group were as follows: control 6 M and 6 F, isoflurane 4 M and 8 F, sevoflurane 4 M and 8 F, and desflurane 4 M and 8 F. The same cohorts of animals were used at both time points.

We performed behavioral testing using the MWM, which has been used in many prior studies (Jevtovic-Todorovic *et al.*, 2003; Liang *et al.*, 2010; Shih *et al.*, 2012; Stratmann *et al.*, 2009a,b) for evaluation of hippocampal-dependent learning and memory. The MWM testing was performed as previously described (Vorhees and Williams, 2006), with minor modifications. The pool was 150 cm in diameter, the platform was 15 cm in diameter, the water was deep enough to cover the platform by 1–2 cm, and the water temperature was kept at 24°C ±2°C. The water was opacified with a small amount of titanium oxide and nontoxic blue paint in order to provide contrast to the white rats, and the sides of the pool were labeled with the cardinal directions (north, south, east, and west) in order to define locations for the platform and for placing animals into the pool. The testing area was equipped with a video camera mounted above the pool and WaterMaze3 imaging software (Coulbourn Instruments, Whitehall, Pennsylvania), which recorded the animals' swim path length, duration (escape latency), and swim speed. After each trial in the pool, the animals were placed in a heated recovery chamber and then returned to their home cages.

Cued training was conducted first, with a white curtain surrounding the pool, consisting of four trials per day for 5 days, and served the purposes of training the rats to swim to a flagged platform and controlling for visual and motor impairments. Then place trials were performed, also consisting of four trials per day for 5 days, in order to measure spatial reference memory. Twenty-four hours after the fifth day of place trials, a 60-s probe trial was conducted to test memory retention.

Statistical analysis. Mortality was compared among groups using χ^2 contingency table analysis. ABG data were analyzed using the Kruskal–Wallis test for nonparametric data (one-way ANOVA) with Dunn's multiple comparison test for each of the three blood gases (Table 1). For the BrdU-positive cell counts, two-way ANOVAs with Sidak's multiple comparison tests were performed to determine differences between anesthetic exposure groups in the three defined regions of the dentate gyrus (Figs. 2A–C). The caspase-3 cell counts (Figure 3) and the MWM swim speeds (Figs. 4D and 5D) were analyzed using the Kruskal–Wallis ANOVA with Dunn's multiple comparisons tests, and the two-way ANOVA with Sidak's multiple comparisons test was used for the MWM probe data (Figs. 4C and 5C). Statistical analysis for the MWM escape latencies (Figs. 4A and B and 5A and B) used a two-way repeated measures ANOVA with

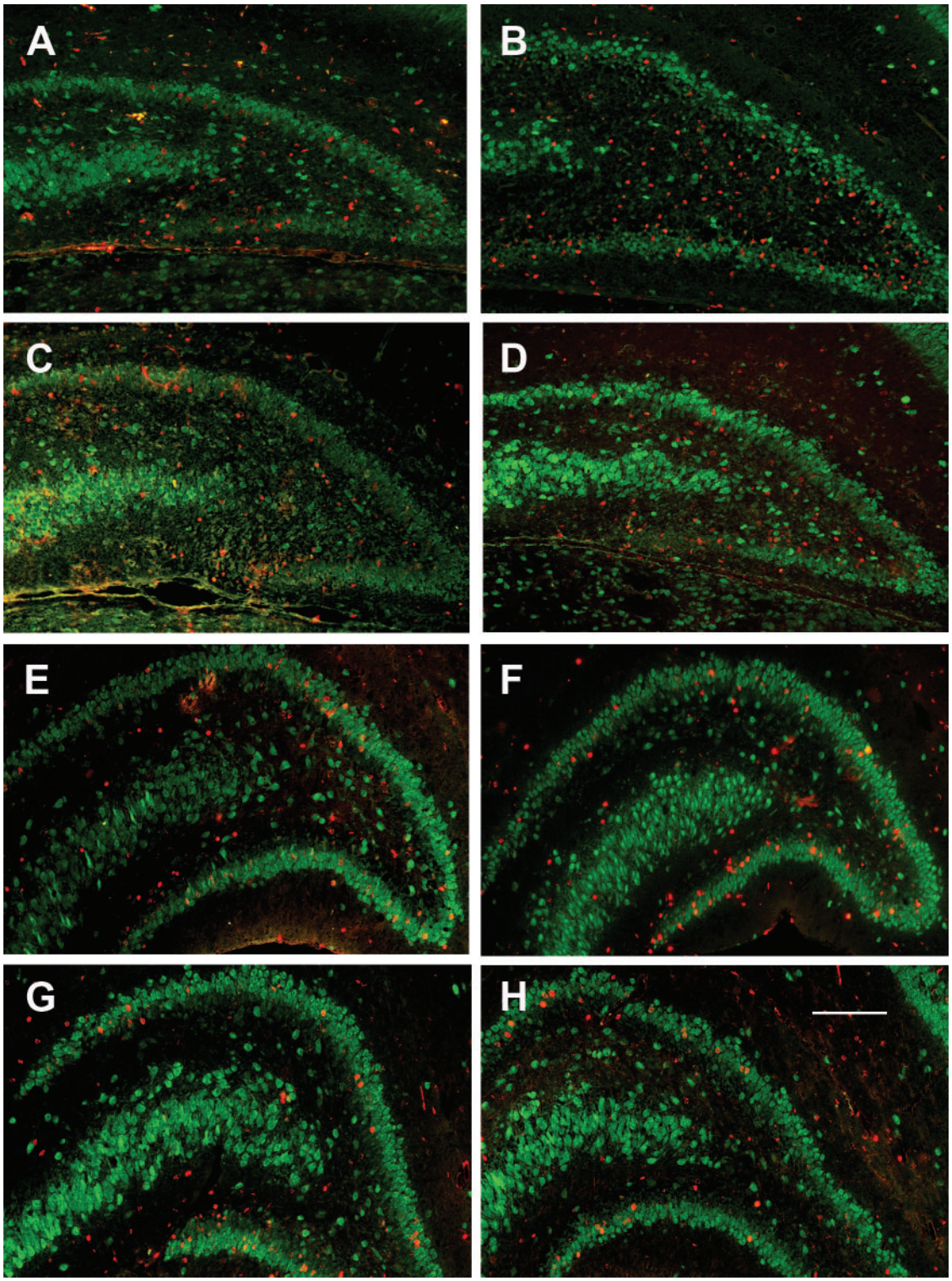

FIG. 1. Immunohistochemistry for BrdU positive cells in the dentate gyrus. A–D, Representative images at P7 showing BrdU labeled cells (red) and NeuN labeled cells (green) for control (A), isoflurane (B), sevoflurane (C), and desflurane (D). E–H, Representative images at P14 showing BrdU labeled cells (red) and NeuN labeled cells (green) for control (E), isoflurane (F), sevoflurane (G), and desflurane (H). Scale bar, 50 microns.

Bonferroni multiple comparisons tests, and nonlinear regression was performed fitting the data to a third order polynomial equation to compare the shape of the curve associated with each anesthetic. Exact *P* values adjusted for multiple corrections are reported in the "Results" section and Table 1. In the figures, the *P* values are reported as inequalities, with $P < .05$ considered significant. The data were expressed as the means ± SEM and statistical analyses were performed using GraphPad Prism v6.0 software (GraphPad Software, Inc, La Jolla, California).

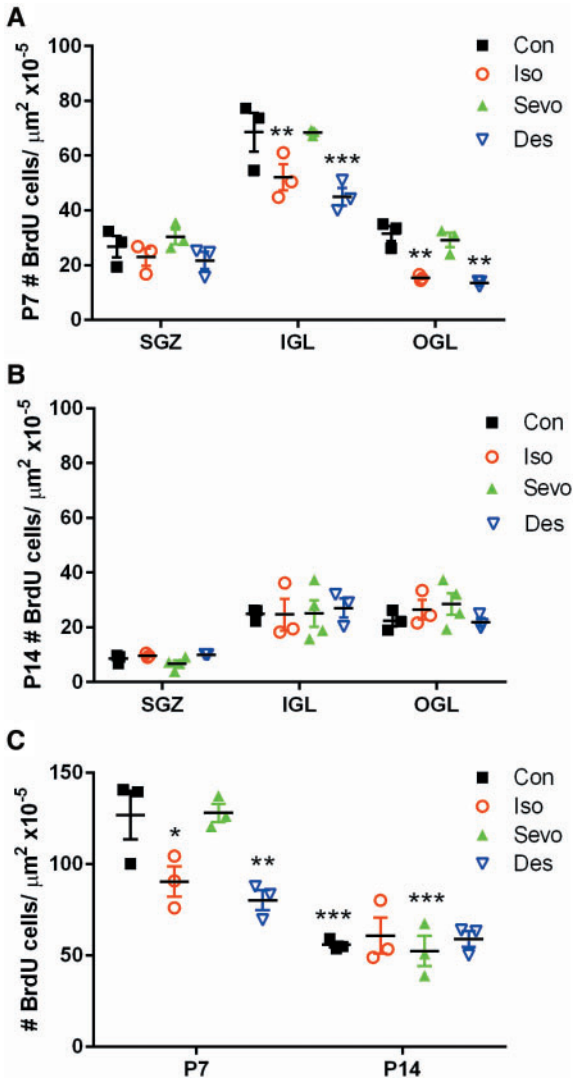

FIG. 2. Quantitative analysis of BrdU-positive cell counts in the dentate gyrus. Number of BrdU positive cells per μm^2 in the subgranular zone (SGZ), inner granular layer (IGL), and outer granular layer (OGL) of the hippocampus. A, At P7, significantly fewer BrdU cells were located in the IGL and OGL in the isoflurane and desflurane exposed groups compared with controls (**$P < .01$, ***$P < .001$). B, At P14, no differences were detected. C, Comparison of total BrdU-positive cell count including all areas at P7 showed significantly fewer BrdU cells at P7 in the isoflurane and desflurane groups (*$P < .05$, **$P < .01$). Total BrdU-positive cell counts including all areas at P14 were equivalent. When comparing P7 to P14 for each anesthetic, there were significantly fewer total BrdU cells including all areas in the control and sevoflurane groups at P14 compared with P7 (***$P < .001$). Data are expressed as means ± SEM. (Con = control, Iso = isoflurane, Sevo = sevoflurane, Des = desflurane; $n = 3$ for each group).

RESULTS

Fate of Neurons Exposed to Anesthetics on Postnatal Day 2

At P7 and P14, we examined the number of cells per area in the SGZ and the inner and outer halves of the granule cell layer of exposed animals compared with controls. At P7, we saw significantly fewer BrdU-positive cells in the inner granular layer (IGL) and the outer granular layer (OGL) of the dentate gyrus for both isoflurane- ($P = .0068$ and $P = .0078$, respectively) and desflurane- ($P = .0002$ and $P = .0028$, respectively) exposed rats compared with controls in the *post hoc* analysis (Figure 2A). There were no significant differences in the number of BrdU-positive cells seen in the IGL or OGL in the sevoflurane-exposed rats compared with controls ($P > .9999$, $P = .9482$, respectively) (Figure 2A). Furthermore, there were no differences in the number of BrdU-positive cells in the SGZ between controls and isoflurane ($P = .8210$), sevoflurane ($P = .8426$), or desflurane ($P = .6702$) (Figure 2A). At P14, there were no significant differences between cell counts in the IGL ($P > .9999$, $P > .9999$, $P = .9560$), OGL ($P = .7834$, $P = .4490$, $P = .9994$), and SGZ ($P = .9932$, $P = .9691$, $P = .9874$) for isoflurane, sevoflurane and desflurane (respectively) compared with controls (Figure 2B).

When we analyzed the total number of BrdU-positive cells in all areas of the dentate at P7 and P14, we found a significant interaction ($P = .0046$). At P7, multiple comparison tests showed statistically fewer BrdU-positive cells after exposure to isoflurane ($P = .0134$) and desflurane ($P = .0019$) compared with controls (Figure 2C), but no difference in cell counts after sevoflurane exposure ($P = .9994$). At P14, there were no significant differences between total BrdU-positive cells in the dentate for all anesthetics compared with controls (isoflurane $P = .9607$, sevoflurane $P = .9854$, desflurane $P = .9898$) (Figure 2C). In addition, there were significantly fewer BrdU-positive cells at P14 compared with P7 for the control and sevoflurane groups ($P = .0005$ and $P = .0003$, respectively), but not for the isoflurane and desflurane groups ($P = .0807$ and $P = .2644$, respectively) (Figure 2C).

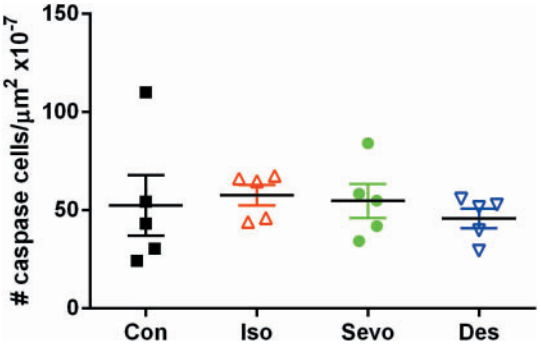

FIG. 3. Cleaved caspase-3 immunohistochemistry at P2 in the dentate gyrus. The number of cleaved caspase-3 positive cells/area was not significantly different in the anesthetic groups compared with controls. Data are expressed as means ± SEM. (Con = control, Iso = isoflurane, Sevo = sevoflurane, Des = desflurane; $n = 5$ for each group).

Cell Death After Anesthetic Exposures on Postnatal Day 2

There were no significant differences detected in the number of activated caspase-3 cells/area between the isoflurane ($P = .5443$), sevoflurane ($P > .9999$), or desflurane ($P > .9999$) exposed groups and controls (Figure 3) in the dentate gyrus at P2.

Long-Term Cognitive Effects of an Anesthetic Exposure on Postnatal Day 2

At 6 weeks post-exposure, there was no significant interaction between treatment groups and controls for the cued training ($P = .3064$) (Figure 4A). This implies that all animals were able to visually locate the platform and learn to "escape" from the pool by reaching the platform. During the place trials, in which the animals must find a hidden platform, a significant interaction was detected in the escape latencies between the treatment groups and controls ($P < .0001$) (Figure 4B). Nonlinear regression analysis for each anesthetic showed that only the isoflurane group was significantly different from controls ($P < .001$). Multiple comparison tests showed that rats exposed to isoflurane or desflurane took significantly longer ($P < .0001$ and

$P = .0002$, respectively) to reach the platform on day three of testing (Figure 4B), but that all animals generally learned to escape from the pool with the same latency by day 5. Twenty-four hours after the fifth day of place trials, the probe trial was performed with the expectation that the rats would spend more time in the target quadrant searching for the platform. No significant interaction was detected in quadrant time and anesthetic exposure ($P = .0793$) (Figure 4C) and *post hoc* analyses also found no significant differences between anesthetic exposures and time spent in each quadrant. The swim speed of the animals, calculated during the probe trial, showed that the desflurane-treated animals were significantly faster than controls ($P = .0008$), but all other treatment groups were equivalent in swim speed ($P = .0545$ for isoflurane, $P = .8615$ for sevoflurane) (Figure 4D).

The MWM testing was repeated at 6 months after the exposures. There was no significant interaction between treatment groups during the cued training ($P = .1957$) (Figure 5A), or during the place trials ($P = .4694$) (Figure 5B). Twenty-four hours after the last place trials, the probe trial was performed, and there were no significant differences between anesthetic exposures and time spent in each quadrant ($P = .0710$) (Figure 5C). *Post hoc* analyses also failed to detect any significant differences between anesthetic exposures and time spent in each quadrant. There was no significant interaction between swim speed and the treatment groups compared with controls ($P = .0616$) (Figure 5D).

DISCUSSION

Our data indicate that inhalational anesthetics cause a transient disruption of normal neuronal development in the dentate gyrus (DG) of the hippocampus, but no long-term cognitive impairment was detected using the MWM. Furthermore, not all inhalational anesthetics tested produced this effect, each having its own neuromodulatory profile.

At P7, we found fewer BrdU-labeled cells in the granular layer of the DG after isoflurane and desflurane exposure, but not after sevoflurane exposure. The relative decrease in cell counts at P7 did not appear to be because of anesthetic-induced apoptosis occurring immediately after the exposure, as we saw no differences in caspase-3 staining at P2 between any anesthetic-exposed animals and controls. However, it should be noted that by performing caspase-3 staining immediately following the 2-h anesthetic exposure, the apoptosis cascade may not have reached its peak. Although the peak may indeed come several hours after the anesthetic exposure (Cheng *et al.*, 2015; Konno *et al.*, 2016), others have shown caspase-3 activation immediately after a 1-h exposure to isoflurane (2%) in the P7 mouse retina (Cheng *et al.*, 2015), or immediately after a 2-h exposure to a cocktail of midazolam, nitrous oxide (75%), and isoflurane (0.75%) in the P7 rat thalamus (Yon *et al.*, 2005). Therefore, if apoptosis were explaining the differences in BrdU cell counts at P7, we would likely have detected it immediately after the exposure at P2.

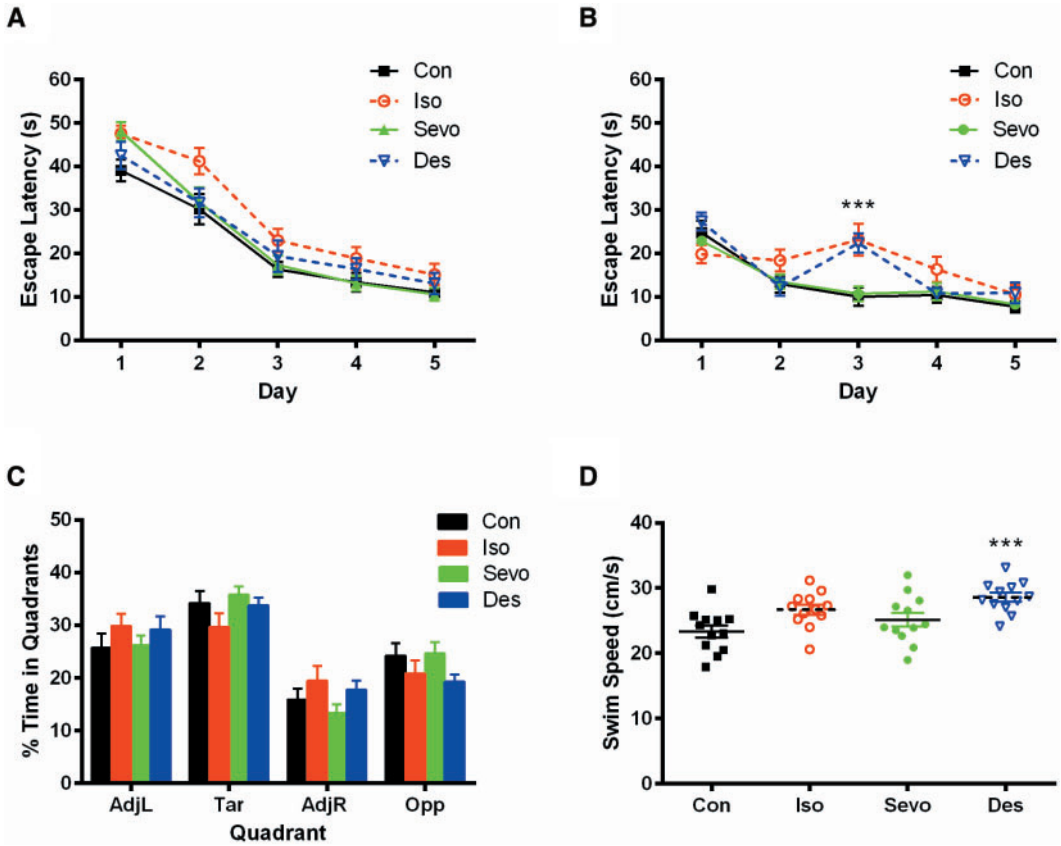

FIG. 4. Morris water maze results at 6 weeks after anesthetic exposure. A, Cued training showed no significant interaction in escape latencies. B, Place trials showed a significant interaction of anesthetic exposure on the learning curve, and nonlinear regression analysis confirmed this effect with isoflurane exposure. *Post hoc* analyses also detected significantly slower escape latencies on day 3 for both the isoflurane and desflurane groups (***$P < .001$). C, No significant differences in the anesthetic exposed rats compared with controls were detected for the probe test. (quadrants: AdjL, adjacent left; Tar, target; AdjR, adjacent right; Opp, opposite). D, Swim speed was significantly faster in the desflurane group (***$P < .001$). (Con = control, Iso = isoflurane, Sevo = sevoflurane, Des = desflurane.) Data are expressed as means ± SEM; $n = 12$ for each group, except in 5C, where Con $n = 11$ because of a technical error.

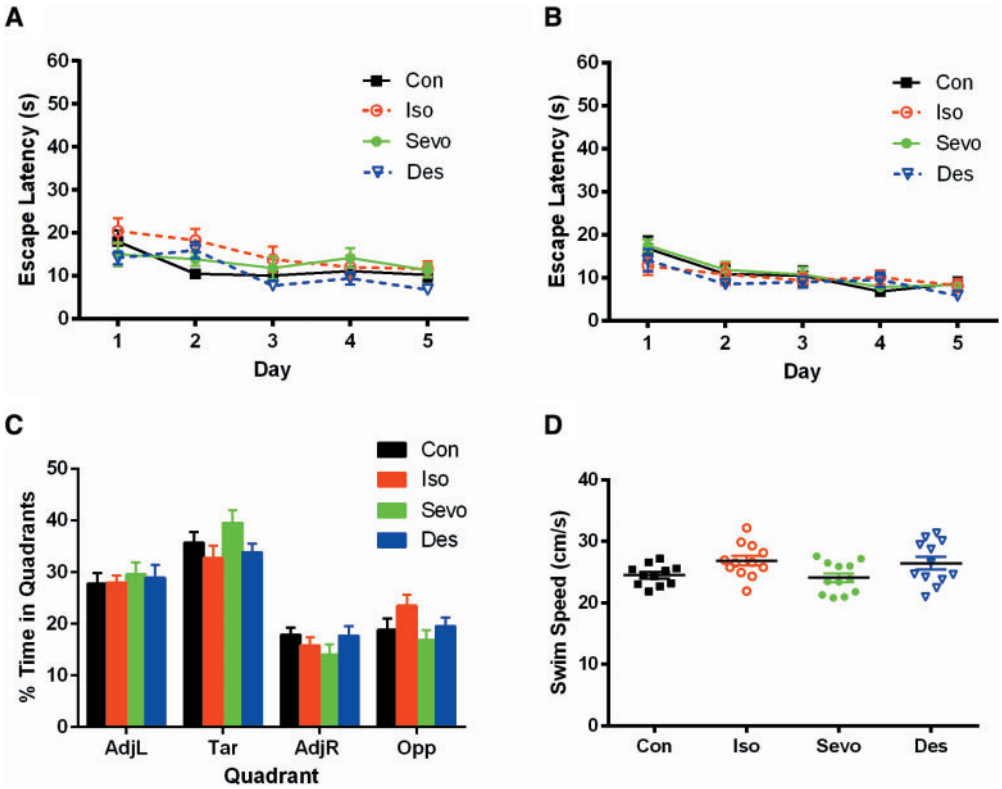

FIG. 5. Morris water maze results at 6 months after anesthetic exposure. There was no significant interaction between anesthetic groups and controls for the (A) escape latencies for the cued training trials, (B) escape latencies for place trials, (C) probe test, or (D) swim speeds. Data are expressed as means ± SEM; $n = 12$ for each group. (Con = control, Iso = isoflurane, Sevo = sevoflurane, Des = desflurane.).

At P14, all groups were comparable but had significantly lower BrdU counts than at P7. This suggests that, in contrast to the P2–7 interval, where proliferation and migration were primarily responsible for the relatively higher cell counts, changes during the P7–14 interval were primarily due to normal developmental apoptosis. It is interesting to note that these normal mechanisms appeared to compensate for the early anesthetic-induced changes in proliferation and/or migration in the isoflurane and desflurane groups.

Our immunohistochemical results suggest that isoflurane and desflurane alter neuronal development by decreasing the number of neurons that have reached the granular layer of the DG by P7. This change could be because of anesthetic effects on cell proliferation, division, or migration. If it were an isolated effect on migration, we would have expected the BrdU-labeled cells in the isoflurane and desflurane groups to be increased at P7 in the SGZ, where these neurons are "born." However, this did not occur, as all anesthetic groups and controls had similar SGZ cell counts. An alternate explanation is that anesthetic exposure caused the BrdU-labeled neurons in the SGZ to have both impaired division and impaired migration to the granular layer. We know that neurons in the DG have the ability to continue to divide into adulthood (Mathews *et al.*, 2010), and that BrdU incorporated during DNA synthesis is passed down to daughter cells in subsequent divisions (Wojtowicz and Kee, 2006). Therefore, we interpret our data to mean that BrdU-positive progenitor cells were transiently delayed in the SGZ at P7, but that these cells regained the ability to divide and migrate between P7 and P14, resulting in the normal number of neurons in the granular layer at P14. The fact that cell counts at P14 are the same across all conditions supports the hypothesis that the developing brain has the ability to compensate for at least some anesthetic-induced developmental stress.

Several studies support the theory that plasticity in the hippocampal DG may compensate for neuronal injury. The SGZ of the DG is an ongoing source of neurogenesis, even in adulthood, presumably providing neuroplasticity to the hippocampus (Drew *et al.*, 2013; Stratmann *et al.*, 2009a, 2014). For example, neurogenesis in the developing DG is increased by brain insults which include cerebral ischemia (Kim *et al.*, 2015), traumatic brain injury (Sun *et al.*, 2015), and early life stress (Naninck *et al.*, 2015). However, other insults, including ethanol exposure (Broadwater *et al.*, 2014), radiation exposure (Roughton *et al.*, 2012), and a 4-h isoflurane exposure (Stratmann *et al.*, 2009b), decreased neurogenesis in the developing DG. This 4-h exposure to isoflurane also impaired adult spatial learning and memory in the MWM (Stratmann *et al.*, 2009b). The fact that we detected only a transient decrease in BrdU-positive cell numbers in the DG and no cognitive deficit may therefore be because of a shorter, 2-h exposure, which presumably allowed neurogenesis to resume. Taken together, these data suggest that ongoing DG neurogenesis may be a compensatory mechanism for early anesthetic-induced changes.

We found that different anesthetics can have distinct neuromodulatory profiles. For example, isoflurane and desflurane caused a transient impairment of neuronal development in the hippocampus, whereas sevoflurane did not. Interestingly, isoflurane and desflurane belong to the same methyl–ethyl ether chemotype (with only one atom difference), whereas sevoflurane is a methyl–isopropyl ether. Others have also detected differences between sevoflurane and desflurane (Shen *et al.*, 2013), and between sevoflurane and isoflurane (Liang *et al.*, 2010).

Support for distinct mechanistic differences between these otherwise similar drugs is evident in recent *in vitro* work that revealed a novel activation of voltage-gated K^+ channels by sevoflurane, but not by isoflurane or desflurane (Barber *et al.*, 2012).

Although it is difficult to predict the cognitive and behavioral consequences of a transient alteration in neuronal development, it seems most reasonable to anticipate a subtle, if any, effect. Accordingly, we detected small but significant changes in spatial learning curves 6 weeks after isoflurane exposure, as well as an impairment on day 3 of the MWM testing after isoflurane and desflurane exposure. However, there were no significant changes in MWM performance at 6 months after anesthetic exposure. The long-term behavioral results for all three anesthetics are consistent with those of another recent study, in which a 30-min sevoflurane exposure on P7 or P15 was not found to result in memory deficits at 3 months post-exposure (Qiu *et al.*, 2016). Our results support the idea that subtle developmental changes induced by anesthetic exposure may not result in detectable long-term neurobehavioral consequences.

Many human retrospective studies have suggested that multiple anesthetic exposures may result in subsequent cognitive dysfunction, and this has been reproduced in some animal studies (Shen *et al.*, 2013; Zhu *et al.*, 2010). When P14 rodents were exposed to isoflurane for 35 min for four consecutive days, they showed a memory deficit as well as fewer hippocampal stem and granule cells (Zhu *et al.*, 2010). In another study, P6 rodents exposed to 3% sevoflurane for 2 h on three consecutive days developed cognitive impairment and increased levels of neuroinflammatory markers, but a single 2-h exposure had no such effects (Shen *et al.*, 2013). The same study also found no significant effects on cognition after a single 2 h or multiple exposures to 9% desflurane (Shen *et al.*, 2013). Another study examined the effects of exposing P7 rat pups to a single 1.8% isoflurane exposure for 2 h compared with three 2-h exposures, 3 days apart. They found that the rats exposed to three 2-h exposures had cognitive deficits at 3 months of age (Murphy and Baxter, 2013). Thus, these studies conclude that a single short exposure does not typically cause long-term damage. The changes seen in neuronal development after either single or multiple anesthetic exposures in animals could be related to the total MAC-hours of exposure, or to accumulated physiological derangements in rodent studies that were sub-optimally monitored and poorly controlled.

The recently published results of two human clinical trials also support our results. The ongoing GAS study, which compared neurodevelopmental outcomes in infants undergoing inguinal herniorrhaphy who were randomly assigned to receive awake-regional anesthesia or general anesthesia using sevoflurane, found no differences in neurodevelopmental outcome at 2 years of age (Davidson *et al.*, 2016). The PANDA study, which compared the IQ scores of children exposed to general anesthesia during inguinal herniorrhaphy under age 3 years with the IQ scores of an unexposed sibling, also found that a single exposure to general anesthesia resulted in no differences in IQ scores later in childhood (Sun *et al.*, 2016).

Our study has several limitations. First, both the control and experimental animals were separated from their mothers during the exposure, which is common practice and considered a control for maternal separation (Jevtovic-Todorovic *et al.*, 2003; Liang *et al.*, 2010; Murphy and Baxter, 2013; Peng *et al.*, 2014; Qiu *et al.*, 2016; Sanchez *et al.*, 2011; Shen *et al.*, 2013; Shih *et al.*, 2012; Stratmann *et al.*, 2009a,b; Yang *et al.*, 2014). However, it has recently been proposed that maternal separation may be more stressful for the conscious control animals than for the anesthetized experimental group, possibly creating long-term behavioral changes in the control animals, primates in particular (Raper *et al.*, 2016). Given that there was no significant difference between the control and experimental groups, the effect is probably subtle in our study. Secondly, although our postnatal day 2 rat pups were not physiologically monitored by objective invasive measures, the similarity between the groups suggests that any untoward physiological derangements did not have had large overall effects on our results. Furthermore, blind cardiac puncture for arterial collection is unreliable, and other means of obtaining arterial blood should be explored in such young rat pups. Finally, our rat pups were sacrificed immediately after the exposures and not enough time may have elapsed to detect the maximum amount of apoptosis at P2.

In conclusion, our results indicate that a single 2-h exposure at P2 to isoflurane, desflurane, or sevoflurane yields no long-term behavioral deficits as evidenced by MWM performance. Isoflurane and desflurane, but not sevoflurane, transiently disrupt brain development and subsequent cognitive function. However, further investigation is warranted to identify the exact mechanisms underlying this disruption. While these data indicate reversibility and an absence of cognitive consequences after a single exposure to one of three volatile anesthetics, future studies are needed to discover the threshold dose and duration at which the neuromodulatory effect becomes irreversible for each anesthetic. Children will continue to require general anesthesia for surgery and procedures, so we must determine the safest possible way to provide it.

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